Isolation and molecular confirmation of *Mycoplasma synoviae* infection from broiler breeder farms in Tamilnadu

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**ABSTRACT**

The presence of pathogenic mycoplasma species in the population of respiratory disease suspected broiler breeder birds in two commercial poultry farms of Tamil Nadu was investigated. A total of 144 samples were collected from suspected rearing breeder birds and examined by culture and species-specific polymerase chain reaction (PCR) assay targeting 16S rRNA of *Mycoplasma synoviae* (MS). 28 samples were found unfit due to high growth of contaminants and hence discarded. The samples showing whirlpool growth in liquid medium (suggestive of mycoplasma) were then inoculated on to solid medium and incubated anaerobically. 42 out of 116 (36.2%) samples showed whirlpool growth and were streaked on to solid medium, however “fried egg” like micro colonies of mycoplasma was evidenced in only 18 samples (15.5%) on day 5 after inoculation. The samples left after discarding the contaminated tubes were subjected to PCR with species specific primer of *M. Synoviae*. 57 out of 116 samples (49.1%) showed positivity to 207 bp (16s rRNA) product of MS. The nucleotide analysis of amplicon showed 96% homology with reference strain by BLAST analysis. This study revealed that MS infection represents a very serious issue to be considered in rearing broiler breeder birds.

**Key words:** Broiler breeder, *M. Synoviae*, Micro-colonies, PCR, Respiratory disease.

**INTRODUCTION**

Poultry industry is one of the leading sub-sectors within livestock sector in India. Tamil Nadu ranks second in India followed by Andhra Pradesh in possessing large scale modern poultry farms (FAO, 2008). Avian mycoplasmosis is one of the major problems in the poultry industry worldwide causing considerable economic losses (Kleven, 2003; Ley, 2003). *Mycoplasma synoviae* (MS) is one of the most important pathogenic mycoplasmas of chicken. MS was first noticed as an agent of infectious synovitis but also played a vital role in causing respiratory disorders (Sato, 1976; Yoder, 1984). Infection can be associated with upper respiratory disease, airsacculitis, synovitis, tenosynovitis, and bursitis. Respiratory complications of MS were higher when found as mixed infection with Newcastle disease, Infectious bronchitis or both (Sato, 1996; Seifi and Shirzad, 2012). The prevalence of MS infection was recorded highest (47.8%) in above 60 weeks of age and lowest (6% - 14.2%) in about 20 weeks of age in rearing breeders (Feberwee *et al.* 2008; Seifi and Shirzad, 2012).

Poultry farms infected with MS suffer huge financial losses due to reduced weight gain, poor feed conversion ratio, culling of lame birds due to synovitis, tremendous drop (20-30%) in egg production, egg shell abnormalities and mortality (Hopkins and Yoder, 1982; Mohammed *et al.* 1987; Feberwee *et al.* 2007; Kleven and Noel, 2008). MS infections vary in infectivity and virulence and infections may sometimes be inapparent (Bradbury, 2001; OIE, 2008). The frequent occurrence of clinical signs suggesting respiratory disease among rearing breeding stock of two leading commercial broiler poultry farms in Tamil Nadu had encouraged us to undertake this study. The diagnosis of avian pathogenic mycoplasmas has typically been carried out by culture, serology and/or molecular-based assays (PCR, deoxyribonucleic acid [DNA] probe). The aim of this study was to isolate mycoplasma in broth and agar culture using specific media from clinical samples collected and then to confirm them by PCR by targeting 16s rRNA sequence using species-specific established primers.

**MATERIALS AND METHODS**

**Culture medium of Mycoplasma:** Sterile swab samples from Choanal cleft and oropharynx were taken from live birds, while from dead birds samples of trachea, lungs and air sacs were collected in PPLO broth (Himedia) contained in a 10ml capacity sterile plastic screw cap tubes. This specific medium

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was supplemented with filter sterilized 0.01% w/v Nicotine Adenine Dinucleotide (Olson and Meadows, 1972), 10% pooled swine yeast serum, 0.01% w/v L-Cysteine HCl and 10% fresh yeast extract. Cysteine HCl is added to reduce the NAD, which is required for the growth of MS. 2.5ml of 10% thallium acetate was added per liter of medium to inhibit the growth of other fastidious bacteria and phenol red was used as pH indicator in the medium.

**Test samples:** Rearing broiler breeding stock (18-20 weeks of age) of two commercial broiler poultry farms in Namakkal district of Tamil Nadu was taken up for study. A total of 144 samples (135 from live birds and 9 from dead carcass) were employed in this study. Distributions of samples in two farms under study were shown in Table 1. Sterile swabs in wooden sticks (HiMedia) were used to collect clinical samples. Choanal cleft and oropharyngeal swabs were taken from live birds and from dead birds samples of trachea, lungs and air sacs were collected.

**Mycoplasma isolation:** All the samples were collected directly into the tubes containing 5ml of medium and closed immediately to minimize the contamination. The culture tubes with swabs and tissue samples were stirred using orbital shaker before incubation. The swabs were left for a day in media to facilitate recovery of organisms and removed later. The tubes were incubated at 37°C, with 90% relative humidity (using cotton wool soaked in water) and 5% CO₂ for 5 to 7 days or until the medium changes from red to yellow. The tubes were examined daily for characteristic whirlpool growth of mycoplasma by shaking the tubes. The tubes suggesting positive for mycoplasma were then streaked onto PPLO agar plates by running water drop technique (Whitford *et al.* 1994). The plates were then incubated anaerobically in desiccator. The plates were examined daily for appearance of mycoplasma colonies under microscope.

**Polymerase chain reaction:** The samples left, after discarding the contaminated tubes were subjected to PCR. The DNA was extracted from the broth culture using DNA extraction kit (Merck India). To confirm the isolate as MS, species specific published primer pair for 16s RNA of MS (Lauerman *et al.* 1993) was used. The sequence for forward and reverse primer used was 5’-GAA GCAA TAG TGA TAT CA-3’ and 5’-GTC GTC TCC TCC GAA GTT AAC AA-3’ respectively. The PCR was carried out as 50 µl volume reaction and in each reaction mixture 2 µl of template DNA was added to 5 µl of 10× PCR buffer, 1 µl 10 mM dNTPs, 20 pmole of forward and reverse primer, 4 units of Taq DNA polymerase and final volume was made to 50 µl using nuclease free water. For DNA amplification the thermal cycler was programmed so as to have initial denaturation at 94°C for 5 mins followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 mins and 1 cycle of final extension at 72°C for 7 mins (Marois *et al.* 2000). The amplified DNA was checked for presence of 207 bp product specific for MS by electrophoresing in 2% agarose gel. 100 bp ladder was used as marker and Ethidium bromide as tracing dye under UV illuminator. Purified PCR products in two pooled samples were sent for sequencing in a reputed laboratory and the sequence data collected was compared with other sequences from Gene bank by BLAST analysis.

**RESULTS AND DISCUSSION**

Among 144 test samples collected, 28 were found unfit due to high growth of contaminants and hence discarded. 42 out of 116 (36.2%) samples showed typical whirlpool growth suggesting mycoplasma, which includes 4 samples from dead birds. On solid medium “fried egg” like raised, round micro colonies with centers was evidenced only in 18 plates (15.5%) from day 5 after inoculation as shown in Fig 1.

**TABLE 1:** Distribution of samples in the two farms in which M. synoviae infection was detected

<table>
<thead>
<tr>
<th>FARM</th>
<th>FLOCKS</th>
<th>NO. OF SAMPLES COLLECTED</th>
<th>NO OF POSITIVE SAMPLES</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>IN BROTH CULTURE</td>
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<tr>
<td>I</td>
<td>A</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>16</td>
<td>NIL</td>
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<tr>
<td></td>
<td>C</td>
<td>22</td>
<td>6</td>
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<tr>
<td></td>
<td>D</td>
<td>10</td>
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<tr>
<td>II</td>
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<td></td>
<td>C</td>
<td>15</td>
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<tr>
<td></td>
<td>D</td>
<td>19</td>
<td>7</td>
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<td></td>
<td>E</td>
<td>13</td>
<td>6</td>
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<tr>
<td>TOTAL</td>
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<td>144-28*= 116</td>
<td>42</td>
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PERCENTAGE OF POSITIVITY

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<tr>
<td>36.2%</td>
<td>15.5%</td>
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<tr>
<td>49.1%</td>
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</table>

*28 samples found unfit for further examination due to heavy growth of contaminants.
From a total of 116 samples screened by PCR, 57 (49.1%) showed positivity to 207 bp product of MS (Marois et al. 2000; Saritha et al. 2010). A typical PCR banding pattern is given in Figure 2 showing 207 bp amplicon of MS. BLAST analysis of nucleotide sequence of amplicon with reference strain from Gene bank showed 96% homology. 100% homology was detected between sequence data of the two sequenced samples.

During this investigation it was found that in the areas surrounding these farms there have been reported a high seroprevalence of avian mycoplasmosis which may be due to overgrowth of many farms in small geographical areas as reported by Kleven and Noel (2008). In the present study inconsistent results were obtained when compared between liquid and solid medium. In some studies it was shown that this could be overcome by applying filtration (0.45 µm) when sub culturing from broth to agar. The filtration method was not used in the current study. All the samples with typical fried egg colonies on solid medium were detected positive for MS by PCR. The PCR analysis of the 24 broth samples from which no growth was observed in the solid media produced positive results and 15 samples which showed no growth in broth were detected positive by PCR. The higher percentage of positivity as detected by PCR in this study is not surprising, as specific PCR was not inhibited by the interference of other bacteria. Detection of mycoplasma by PCR has been proven to be a very specific and sensitive which can amplify even very small amount of nucleotides that cannot be easily detected by other methods (Nascimento et al. 1991; Ramdass et al. 2006; Saritha et al. 2010). Results from this study indicated that 16s RNA gene amplification could be used for confirmation of MS from field samples (Lauerman et al. 1995; Kemf, 1996). The PCR assay used here facilitated identification of mycoplasma agents even from contaminated broth samples. The 100% homology between the sequenced samples may be due to same source of infection because the disease could be transmitted both horizontally and vertically and remain in the flock constantly as subclinical infection (Bradbury and Jordan, 2003). This study detected highest seroprevalence (49.1%) of MS infection among rearing breeding stock which was in contrary with the findings of other research groups (Feberwee et al. 2008; Seifi and Shirzad, 2012). Analysis from farm details showed that this highest prevalence may be due to replacement of the breeding stock by the progeny of the same stock. The results of the current study especially revealed that mycoplasma synoviae infection represent a serious issue to be considered even in rearing broiler breeders.

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REFERENCES


